Entropy-driven Mg^{2+}-induced folding of TPP riboswitch RNA

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ABSTRACT

Mg^{2+} is well-known to facilitate the structural folding of RNA. However, the thermodynamic and dynamic roles of Mg^{2+} in RNA folding remain elusive. Here, we exploit smFRET (single-molecule fluorescence resonance energy transfer) and ITC (isothermal titration calorimetry) to study the mechanism of Mg^{2+} in facilitating the folding of TPP (thiamine pyrophosphate) riboswitch RNA. The results of smFRET identify that the presence of Mg^{2+} compacts the RNA, but also enlarge the dynamical dispersity among individual RNA molecules, resulting in a large gain of entropy. The compact yet flexible conformations triggered by Mg^{2+} may help the riboswitch to recognize its specific ligand and further fold. This is supported by the ITC experiments, in which the Mg^{2+}-induced RNA folding is driven by entropy (ΔS) instead of enthalpy (ΔH). Our results complement the understanding of Mg^{2+}-induced RNA folding. The strategy developed in this work can be used to model other RNAs' folding at different conditions.

Keywords: Mg^{2+}; Riboswitch RNA; Thermodynamics; Entropy-driven; smFRET; ITC

INTRODUCTION

The existence of counterions contributes to the structural folding and functions of RNA. Magnesium ions (Mg^{2+}) can screen the Coulombic repulsion and stabilize RNA tertiary structures by neutralizing phosphate groups, and has been widely used for facilitating RNA folding (Fiore et al. 2012; Haller et al. 2013; Börner et al. 2016; Manz et al. 2017; St-Pierre et al. 2021). Many studies have been focused on dissecting RNA folding upon the addition of Mg^{2+} (Fiore et al. 2012; Holmstrom et al. 2012; Haller et al. 2013; Manz et al. 2017; White and Hoogstraten 2017; Rode et al. 2018; St-Pierre et al. 2021). However, the thermodynamic roles of Mg^{2+} in RNA folding have been controversial. Based on the second law of thermodynamics, the free energy (ΔG) should be reduced for spontaneous folding of RNA in the presence of Mg^{2+}. As $\Delta G = \Delta H - T\Delta S$, the reduction of ΔG can be achieved either by reducing enthalpy (ΔH) or by increasing entropy (ΔS). As widely believed, Mg^{2+} can alleviate the enhanced Coulombic interaction associated with a more compact RNA (Vander Meulen and Butcher 2012; White and Hoogstraten 2017; Sung and Nesbitt 2019), and it plays an enthalpy-dominated role. On the contrary, some studies support that entropy change is the primary source responsible for stabilizing RNA with Mg^{2+} (Kulshina et al. 2010; Fiore et al. 2012; Kilburn et al. 2016; White and Hoogstraten 2017; Rode et al. 2018). However, there are few reports on the direct thermodynamic characterization of complex RNA folding in the presence of Mg^{2+}, and the detailed mechanism of Mg^{2+}-induced RNA folding has not been clarified.

Riboswitches are *cis*-acting RNA elements that locate at the 5′ untranslated regions of mRNA. Riboswitches can control downstream gene expression by changing their structures after binding to specific ligands (Garst et al. 2011; Lotz and Suess 2018; Bou-Nader and Zhang 2020). A riboswitch typically comprises a ligand-sensing aptamer domain and an expression platform (Garst et al. 2011). The aptamer binds to its specific ligand with high affinity, resulting in conformational change of the expression platform and affecting gene expression (Mandal and Breaker 2004). TPP riboswitch is a

well-characterized riboswitch, and it can regulate the gene expression related to TPP synthesis and thiamine transport in bacteria, archaea, fungi, and plants (Winkler et al. 2002; Kubodera et al. 2003; Sudarsan et al. 2003; Galagan et al. 2005; Yamauchi et al. 2005; Cheah et al. 2007). Here, we exploited smFRET and ITC measurements to dissect the Mg^{2+}-dependent kinetic and thermodynamic properties of the aptamer domain of the TPP riboswitch, riboTPP. smFRET results indicate that riboTPP prefers a more compact structure in the presence of Mg^{2+}. Unexpectedly, the transition frequency between the low-FRET and high-FRET state enhances dramatically with Mg^{2+}. To elucidate the thermodynamic force for the binding between Mg^{2+} and riboTPP, we performed ITC experiments and observed little enthalpy change (ΔH) upon Mg^{2+} addition, and significant entropy change (ΔS) instead. This indicates that Mg^{2+}-triggered riboTPP folding is primarily entropy-driven, instead of enthalpy-driven.

RESULTS

Mg^{2+}-induced Conformational change of riboTPP

TPP riboswitch is the most populated riboswitch discovered in bacteria, fungi, archaea, plants, and eukaryotes (Winkler et al. 2002; Sudarsan et al. 2003; Cheah et al. 2007). The aptamer domain of the TPP riboswitch can also exist in tandem modules (Sudarsan et al. 2006; Welz and Breaker 2007). TPP binds to the riboswitch with a K_d of ~ 50 nM, resulting in translational inhibition of its downstream gene (Nudler 2006; Lotz and Suess 2018). Such a high binding affinity relies on specific recognition of the pyrophosphate moiety and pyrimidine ring of TPP (Serganov et al. 2006). RiboTPP studied here is from *E. coli thiM* (Fig. 1A). It is composed of 88 nucleotides, with the mutations at the ends to facilitate synthesis (Supplemental Table S1). We applied single-molecule FRET to study the conformational dynamics of riboTPP upon Mg^{2+} addition. Based on the crystal structure of riboTPP (Serganov et al. 2006), we introduced Cy3 and Cy5 fluorophores to sites 59 and

39, respectively. The dual-fluorescently labeled riboTPP, Cy3Cy5-riboTPP was hybridized with a biotinylated DNA to immobilize on coverslips for smFRET measurements (Fig. 1B).

The conformational changes of the dual-fluorescently labeled riboTPP were monitored over time by recording the emission intensities of both Cy3 (I_D) and Cy5 (I_A) fluorophores. The apparent FRET efficiency was then calculated as $E_{FRET} = I_A / (I_A + I_D)$ to estimate the temporal changes in distance between two labeled sites at riboTPP (Roy et al. 2008). Mg^{2+} exists in living cells at millimolar concentrations (Börner et al. 2016), and smFRET measurements were performed at 0, 0.5, 2.0, 10.0 and 20.0 mM Mg^{2+} to explore how Mg^{2+} influences dynamics of riboTPP. The representative single-molecule traces from 0.1 to 100 seconds with anti-correlated Cy3 and Cy5 emission were shown (Fig. 2A). The fluorescent intensities of Cy3 and Cy5 were converted into transfer efficiencies (blue lines in Fig. 2A). And the transfer efficiencies (E_{FRET}) fluctuated between a low-FRET (~ 0.4) state, possibly corresponding to the Mg^{2+}-free state, and a high-FRET (~ 0.7) state (likely the folded state). The dynamic behaviors of individual molecules were characterized by hidden Markov modeling analysis (HMM) (Bronson et al. 2009). Three sample traces showed transitions between the low-FRET and high-FRET states of the RNA molecules in the presence of 2.0, 10.0, and 20.0 mM Mg^{2+} (Fig. 2A). The highlighted arrows indicate the photobleaching events of donor or acceptor, occurring at 35.6 s, 47.9 s, 36 s, 48.1 s, 39.3 s, respectively for the respective trajectories in Figure. 2A. The control experiment by using Cy3-labeled riboTPP was conducted to ensure the monitored signals of Cy3Cy5-riboTPP come from the conformational changes instead of photobleaching or blinking of fluorescence or laser fluctuation (Supplemental Fig. S1). For Cy3-labeled riboTPP, neither ensemble nor single-molecule data showed dependence on Mg^{2+}, and the FRET leakage of the system is as low as ~ 0.08 (Supplemental Fig. S1).

Mg^{2+}-induced Conformational Compaction of riboTPP

We further identified the Mg^{2+}-induced folding of riboTPP by depicting efficiency contour plots (Gregorio et al. 2017), which are color-coded from white (lowest) to red (highest) and normalized using more than 5,000 single-molecule FRET traces (Fig. 2B). In the absence of Mg^{2+} (Fig. 2B, top panel), a low-FRET state (~ 0.4) dominated in riboTPP. In contrast, the high-FRET state (FRET ~ 0.7) gradually populated with the increase of Mg^{2+}, and broadly distributed in the presence of 2 mM Mg^{2+} (Fig. 2B, middle). And the high-FRET conformation (~ 0.7) was overwhelmingly dominated at 20 mM Mg^{2+} (Fig. 2B, bottom panel). The results support that the intrinsic structural dynamics and conformational compact of riboTPP with the addition of Mg^{2+} as reported elsewhere (Brenner et al. 2010; Haller et al. 2011; Liberman et al. 2015; Roy et al. 2017).

Ensemble information was attained by assembling smFRET trajectories into population FRET histograms. Two states of riboTPP were identified in the cumulative population histograms (Fig. 2C). In the absence of Mg^{2+}, the FRET histogram of riboTPP showed a dominant distribution of the low-FRET state (~ 89.2%) (Fig. 2C, top panel), indicating the riboTPP adopted two conformations in the absence of Mg^{2+}, as reported elsewhere (Haller et al. 2013). Increasing Mg^{2+} (0.5 ~ 20.0 mM) led to larger contribution of the high-FRET state (Fig. 2C). The presence of Mg^{2+} ions resulted in lessening of the low-FRET state from 89.2% (0 mM Mg^{2+}) to 19.1% (20 mM Mg^{2+}), and reciprocally elevated the abundance of high-FRET states. The low-FRET state (E_{FRET} ~ 0.4) corresponded to a more extended *apo* form of the TPP riboswitch (Fig. 2D, left), in which J3-2, P4, and J2-4 regions were probably in the open conformation (Haller et al. 2013). The high-FRET state (E_{FRET} ~ 0.7) may be associated with a compact structure of riboTPP bound with Mg^{2+} (Fig. 2D, right) (Baird and Ferré-D'Amaré 2010; Haller et al. 2013). The mean values of FRET from 0.4 to 0.7 reflected inter-dye distances shortened from approximately 58 Å to 46 Å upon Mg^{2+} addition. The results indicate that the helices P3 and P4 of riboTPP become closer upon the addition of Mg^{2+}, and high Mg^{2+} triggers riboTPP to adopt the folded structure predominately. This is consistent with the common belief that

Mg^{2+} stabilizes RNA to a compact conformation (Brenner et al. 2010; Haller et al. 2011; Liberman et al. 2015; Roy et al. 2017). The fractions of low-FRET and high-FRET states were fitted to Mg^{2+}-dependent curves with Hill coefficients of 2 (Fig. 2E). The calculated half-maximum effective concentration value (EC_{50}) of Mg^{2+} for the high-FRET state is approximately 1 mM (Fig. 2E), similarly as intracellular concentration of Mg^{2+}. Mg^{2+}-induced folding was also reported for other riboswitch RNAs (Brenner et al. 2010; Haller et al. 2011; Liberman et al. 2015; Roy et al. 2017). For riboTPP, there are the existence of two conformations with and without Mg^{2+}, and conformational compaction is observed in the presence of Mg^{2+}. This is consistent with the conformational selection mechanism (Ye et al. 2016; Rode et al. 2018).

Mg^{2+}-induced Conformational Transition of riboTPP

The addition of Mg^{2+} affected fluctuations rates between the high- and low-FRET states in riboTPP (Fig. 2A). According to their interconversion frequencies, we classified the molecules into (i) static (i.e., no transition within observation time), and (ii) dynamic. The dynamic molecules were informative ones, which were characterized using a two-state HMM and transition density plot (TDP) to explore the conformational switches between states (McKinney et al. 2006; Liauw et al. 2021). HMM analysis and TDP at different Mg^{2+} indicate that these molecules transit from between both high-to-low and low-to-high FRET states at a rate of ~ 0.5 s^{-1} (Fig. 2A,F). In the absence of Mg^{2+}, less than 5% traces switched between the low-FRET and high-FRET state (Fig. 2F, top panel). This indicates that the two states slightly interconverted in 100 s, and the majority of riboTPP display monomeric behavior at the low-FRET state, similarly as reported for other RNAs (Manz et al. 2017; Roy et al. 2017; Manz et al. 2018). In the TDP of 0.5 mM Mg^{2+}, ~11% of riboTPP displayed dynamic transition. And 2.0 and 20.0 mM Mg^{2+} increased the fractions of dynamic riboTPP to ~21% and 27%, respectively (Fig. 2F, bottom). And additionally, the majority of static high-FRET

conformation only appeared with Mg^{2+} as high as 10.0 or 20.0 mM. We then quantified the relative fractions of dynamic riboTPP at different Mg^{2+}, the dynamic riboTPP was sensitive to Mg^{2+} and enhanced greatly as Mg^{2+} increased. Therefore, although the majority of riboTPP stayed static at the observed time window even with saturating Mg^{2+}, Mg^{2+} did increase the dynamics of riboTPP. The results indicate that additional Mg^{2+} ions are preferably associated with the compact conformations resembling the high-FRET state of riboTPP, and simultaneously, favors flexibility and dynamics as reported elsewhere (Steffen et al. 2020).

Thermodynamic measurements of riboTPP upon Mg^{2+} titration

Isothermal titration calorimetry (ITC) can be used to calculate the enthalpy change (ΔH), entropy change (ΔS), Gibbs free energy (ΔG), and dissociation constants (K_d) of binding (Kulshina et al. 2010; Rode et al. 2018; Zeller et al. 2022). Calorimetric characterization of ligand binding has previously been reported for riboTPP (Kulshina et al. 2010; Zeller et al. 2022), but direct examination of thermodynamical parameters of Mg^{2+} binding to riboTPP has not been reported yet. To elucidate the thermodynamic nature of the Mg^{2+}-stabilized riboTPP, we performed ITC to deconvolute the roles of enthalpy and entropy of riboTPP with Mg^{2+} (Fig. 3 and Table 1). A series of binding experiments were conducted by titrating different concentrations of Mg^{2+} to riboTPP. The representative ITC curves were shown in Figure. 3A-C, and the ΔH, ΔS, and ΔG upon the titration of Mg^{2+} to riboTPP at room temperature were illustrated in Figure. 3D. Based on $\Delta G < 0$, the binding between Mg^{2+} and riboTPP was thermodynamically favorable as expected (Fig. 3 and Table 1). However, 20 mM Mg^{2+} binding to riboTPP was entropically favored ($-T\Delta S$ = -4.74 kcal·mol^{-1}) and enthalpically disfavored (ΔH = 0.1 kcal·mol^{-1}), with a K_d of 0.4 mM. 50 mM Mg^{2+} bound to riboTPP with a K_d of 1.2 mM, and its entropic penalty decreased slightly ($-T\Delta S$ = -4.14 kcal·mol^{-1}). Increasing Mg^{2+} to 80 mM had a great impact on K_d (Fig. 3C and Table 1), but slightly affected the

thermodynamical parameters ($-T\Delta S$ = -3.84 kcal·mol^{-1}, ΔH = 0.38 kcal·mol^{-1}). These results show that it is $-T\Delta S$ mainly contributes to the negative ΔG for Mg^{2+} binding to riboTPP, and therefore, Mg^{2+} binding to riboTPP is entropy-driven. This favorable entropy is likely contributed by flexible and dynamical species of riboTPP upon Mg^{2+} addition, consistent with our previous smFRET observations that Mg^{2+} increased flexibility, heterogeneity, and dynamics of riboTPP at single-molecule levels in Figure. 2.

DISCUSSION

Based on single-molecule FRET and ITC measurements, we proposed the model of Mg^{2+} facilitated riboTPP folding (Fig. 4). The model contains three distinct conformations of TPP riboswitch, fully unfold (U), low-FRET (*apo*, A), and high-FRET (folded, F) states. Two states, low-FRET and high-FRET states, are observed at smFRET, and the fully unfolded state is probably too lowly populated or too dynamic to be detected at smFRET. In the absence of Mg^{2+}, riboTPP is considerably less organized, and predominantly at the low-FRET state. In contrast, a propensity of the compact conformation is observed at saturating Mg^{2+} (Fig. 2). This suggests that Mg^{2+} promotes folding of riboTPP. This is supported by the crystal structure, in which Mg^{2+} ions are localized at the aptamer core (Serganov et al. 2006; Thore et al. 2006; Serganov and Nudler 2013). Moreover, our smFRET data unambiguously show structural heterogeneity of riboTPP with high Mg^{2+}, which would facilitate the ligand binds to an amenable structure and access to the core of the RNA. These behaviors are accompanied by a dominant entropy loss (Fig. 3). There must be additional entropy sources beyond simple electrostatic interaction, and Mg^{2+} binding to riboTPP may be accompanied by partial dehydration and non-Coulombic (Paulsen et al. 1988; Kankia and Marky 1999; Kulshina et al. 2010; Kilburn et al. 2016), and replacement of sodium with magnesium in the RNA core may be

observed as a slightly positive ΔH contribution (Krakauer 1972). These observations have implications for how Mg^{2+} binding in the aptamer predisposes the folded conformations and favors ligand recognition. We also hypothesize that such free energy changes result from partially folded RNA interaction with Mg^{2+}, totally different with temperature-induced unfolding measurements mainly contributed by enthalpy, i.e., hydrogen bond, base stacking, and other interactions (Fiore et al. 2012; Kilburn et al. 2016; Sung and Nesbitt 2019). The results we observed here are in good agreement with ΔS for the TPP riboswitch ligand-binding under multiple Mg^{2+} concentrations (Kulshina et al. 2010; Zeller et al. 2022), or ΔS for the temperature-induced RNA unfolding experiments at different Mg^{2+} (Fiore et al. 2012; Kilburn et al. 2016; White and Hoogstraten 2017; Rode et al. 2018). The entropic driven of Mg^{2+} induced folding was also reported for a few simple RNAs (Kulshina et al. 2010; Fiore et al. 2012; Kilburn et al. 2016; White and Hoogstraten 2017; Rode et al. 2018). These previous studies for simpler RNAs together with our results for a more complex riboswitch RNA identify that entropy-driven is a characteristic property of Mg^{2+}-induced RNA folding.

This work explores the single-molecule FRET kinetics and ITC thermodynamics to improve our understanding of the Mg^{2+}-facilitated folding of RNA. Preliminary single-molecule data identify two FRET states, corresponding to two distinct conformations. The low-FRET state shifts to a high-FRET state, indicating Mg^{2+}-induced compaction of riboTPP. Moreover, riboTPP dynamically fluctuates between two states in the presence of Mg^{2+}, indicating an increase of heterogeneity for the RNA upon the addition of magnesium ions. It is worth pointing out that the overwhelming majority of single-molecule remains static for both low-FRET and high-FRET states even with saturating Mg^{2+}. And the free energy barrier reduction for Mg^{2+} binding is overwhelmingly contributed by the entropic part ($-T\Delta S$) rather than the enthalpic component (ΔH). The entropically beneficial effects of increased Mg^{2+} on riboTPP result from heterogeneity and structural flexibility of the RNA as

evidenced in smFRET, resulting in entropy increase. What's more, another entropic effect of Mg^{2+} binding is that a compact yet flexible RNA may help the further recognition and folding of the riboswitch by its specific ligand. The last aspect points to an essential difference between the thermodynamics of RNA folding observed by ITC and traditional temperature-dependent experiments (Fig. 4). Mg^{2+} ions mediated tertiary structures of riboTPP is an entropy-driven process, which is against the common sense that RNA folding is predominately entropy-driven (i.e., $-T\Delta S \ll \Delta H$). This work provides new insight into the thermodynamics associated with the formation of Mg^{2+} induced folding in RNA, and highlights entropically driven mechanisms at the RNA folding step to the Mg^{2+} ion. And our observations are supplemental to the understanding of Mg^{2+}-triggered folding of biological macromolecules.

MATERIALS AND METHODS

RNA Preparation

All the TPP riboswitch in this work was in-house prepared. The coding DNA template used to produce the sample is provided in Supplementary Material, Table S1. The unlabelled TPP riboswitch sample (sequence in Table S1) used in ITC was generated by *in vitro* transcription with T7 polymerase (Liu et al. 2018). The TPP riboswitch labeled with Cy3 and Cy5 used in FRET was produced by PLOR methods (Liu et al. 2015; Liu et al. 2018). All coding and non-coding templates (listed in Table S1) used in RNA synthesis were prepared by PCR, and the non-coding DNA template used in PLOR contained 5'-biotin. The unlabeled RNA products for ITC were purified by denaturing 12% PAGE (polyacrylamide gel electrophoresis). And the fluorescently labeled TPP riboswitch was purified by PAGE and reverse-phase HPLC using a C8 column (#EXL-122-2546U, Advanced Chromatography Technologies Ltd., UK) with multiple-wavelength detection (UV at 260 nm, fluorescence at 550 nm for Cy3 or fluorescence at 650 nm for Cy5). The purified RNA was exchanged to the desired buffer and stored at -20 °C. The unlabeled RNA for ITC experiments was heated at 80 ~ 85 °C for 5 min, and then cooled down to room temperature before use. The labeled RNA for FRET experiments were hybridized to the biotin-labeled DNA linker (Table S1) in T50 buffer (50 mM NaCl, 10 mM Tris-HCl, pH 8.0) by heating at 95 °C for 5 min and then cooling down to room temperature before use.

Single-molecule FRET detection and its data analysis

200 μL, 0.01 mg/mL streptavidin was injected into the chamber with PEG-passivated coverslips and incubated for about 1 min before imaging acquisition started. Then TPP riboswitch was immobilized to microscope flow chamber *via* biotinylated DNA linker (sequence in Table S1) to PEG-passivated coverslips, which were decorated with mPEG-biotin and streptavidin (Fig. 1B). This immobilization scheme has been reported for other systems in studies of their dynamics and

functions (Roy et al. 2009). All the single-molecule FRET measurements were performed at 20 °C in the imaging buffer (50 mM NaCl, 10 mM Tris-HCl, pH 8.0), either in the absence or the presence of 20 mM Mg^{2+}. An enzymatic deoxygenation system composed of 0.8% wt/vol D-glucose, 1 mg/ml glucose oxidase (Sigma-Aldrich), 0.04 mg/ml catalase, 3 mM 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox, Sigma-Aldrich) was used to alleviate the fluorescent photobleaching and blinking (Romo-Uribe 2021).

We used objective-type total internal reflection fluorescence (TRIF) microscopy to perform single-molecule FRET measurements based on an inverted microscope (Eclipse Ti, Nikon) at 20℃. The videos were recorded with an EMCCD camera (Andor iXon Ultra 897) using a 100 ms time resolution and a total length of 100 s. The solid-state 532 nm and 640 nm excitation lasers (OBIS Smart Lasers, Coherent Inc.) are modulated using digital signals from the EMCCD camera. The donor (Cy3) is excited by a continuous wave 532-nm laser. A 1.49 NA 1003 oil immersion lens (Apo TIRF, Nikon, Tokyo) was used to generate an evanescent field for illumination. Fluorescence emission signals from Cy3 and Cy5 were collected by the camera and spectrally separated by a dichroic mirror (T635lpxr, Chroma) in a Dual-View spectral splitter (Photometrics, Inc., Tucson, AZ). The CellVision software (Beijing Coolight Technology) was utilized to control the hardware and record the single-molecule fluorescence trajectories at a frame rate of 10 Hz (Yang et al. 2018).

Preprocessing of single-molecule videos was performed with the open-sourced software package iSMS to extract the time-dependent signal (Preus et al. 2015). Cy3 and Cy5 spots were detected using the intensity threshold of 100, and using a 2-D Gaussian function fitting was used to further identify the fluorescent spots on the EMCCD. The background signal, which was the average intensity of all pixels with a 2-pixel distance to the fluorescent spot, was subtracted. The deepFRET software was used to accurately identify the photobleaching events and single-molecule trajectories (Thomsen et al. 2020). The data used for analysis were truncated before photobleaching. The single-

molecule traces with the anti-correlated behavior between Cy3 and Cy5 intensities were selected for further statistical analysis. The FRET efficiency, E_{FRET}, was directly calculated *via* the following equation: $E_{FRET} = I_A / (I_A + I_D)$ (Roy et al. 2008). Here, I_D and I_A represent donor and acceptor fluorescence intensity, respectively. As E_{FRET} is associated with the inter-dyes distance, the temporal evolution of E_{FRET} was used to monitor the fluctuation of the distance between the two labeled sites. A Förster radius (R_0) of 55 Å was used to convert raw FRET efficiency to an approximate distance using $E_{FRET} = 1/(1 + (R/R_0)^6)$. The idealized fits of FRET efficiency resulted from the hidden-Markov-modeling (HMM) package vbFRET; the vbFRET uses an empirical Bayesian method to estimate the FRET states and the time points at which transitions between FRET states occur (Bronson et al. 2009). Based on the sequence of HMM assigned state to count the transition events, then the transition density plots (TDP) for the single RNA are visualized using Python module matplotlib (https://matplotlib.org). Moreover, Dose-response function $y(x) = A1 + (A2-A1)/(1+10^{(\log Z - x)/P)})$ was used for fitting data points in Figure. 2E to obtain EC50 value (Gregorio et al. 2017), where *A1* is the lower asymptote, *A2* is the upper asymptote, *P* is the Hill slope, and *Z* is the EC_{50}.

Isothermal Titration Calorimetry (ITC) measurements

Isothermal titration calorimetry (ITC) experiments were used to measure the interactions between Mg^{2+} and TPP riboswitch in the buffer containing 50 mM HEPES, 100 mM NaCl, pH 7.5 on a MicroCal ITC_{200} calorimeter (General Electric, USA) at room temperature 25ºC. 2 μL 20 mM Mg^{2+} ions were injected to the sample cuvette containing 200 μL, 20 μM TPP riboswitch for 20 times using an automatic microsyringe with 90 seconds interval. The titration between the buffer and TPP riboswitch was referred to as the background for ITC, which was subtracted from the raw data of ITC. The integration of the fitted data using the one-set-of-sites model in MicroCal Auto-ITC_{200} Origin (OriginLab, USA) gives the binding parameters enthalpy ΔH (kcal·mol^{-1}), association constant K (M^{-1}), and n (bound Mg^{2+} ions per RNA). The binding free energy ΔG and reaction

entropy ΔS can be achieved from the relationships $\Delta G = -RT \ln K$, where R = 1.987 cal·mol^{-1}·K^{-1}, T = 293 K, and $\Delta G = \Delta H - T\Delta S$. The obtained thermodynamic parameters (ΔG, ΔH, ΔS) are illustrated in Figure. 4.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

COMPETING INTEREST STATEMENT

The authors declare that they have no competing interests.

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Figure Legends

FIGURE 1. The secondary structure of riboTPP and experimental setup for smFRET. (*A*) The secondary structure of riboTPP. riboTPP contains five coaxial helical regions (P1 to P5) and two junctions (J2-4 and J3-2). Fluorescent donor (Cy3) and acceptor (Cy5) covalently linked to sites 59 and 39 are depicted as green and red spots, respectively. (*B*) Schematic diagram of single-molecule FRET for riboTPP. The model of riboTPP was adapted from the crystal structure (PDB ID: 2GDI). The RNA was immobilized on PEG-passivated coverslips by a biotinylated DNA (gray), and imaged *via* total internal reflection fluorescence (TIRF) microscopy.

FIGURE 2. Mg^{2+} promotes the folding and flexibility of TPP riboswitch revealed by smFRET. (*A*) Representative single-molecule trajectories of TPP riboswitch at different Mg^{2+} concentrations (0, 0.5, 2.0, 10.0 and 20.0 mM). Observed donor (I_D), acceptor (I_A) intensities, and the FRET efficiency ($E_{FRET} = I_A / (I_A + I_D)$) are shown in green, red, and blue lines, respectively. Arrows indicate photobleaching events of either donor or acceptor. The coarse-grained FRET-state sequences (blacked lines) are derived by hidden Markov modeling (HMM) modeling. (*B*) The FRET contour plots (>2.1 s) are achieved from all the single-molecule FRET traces at different Mg^{2+}. The normalized population contour plots are color-coded from white (lowest) to red (highest). (*C*) Histograms derived from overall smFRET traces show the FRET populations for riboTPP at 0 mM Mg^{2+} (top) to 20 mM Mg^{2+} (bottom). The FRET histograms display two distinct states. Low-FRET (~ 0.4) and high-FRET (~ 0.7) represents Apo form [A] and folded [F] states, respectively. The blue lines indicate the sum of Gaussian fits. (*D*) Schematic diagram of Mg^{2+}-induced folding of riboTPP. (*E*) Mg^{2+} compacts riboTPP. The relative occupancies of low-FRET and high-FRET states are obtained from Gaussian fits. This data was fitted to a dose-response function with Hill coefficient

fixed at 2.0, with 1 mM EC_{50} for Mg^{2+}. (*F*) Transition density plots (TDP) depicting heatmap contour show the dynamic transition of RNA collected at 0 ~ 20 mM Mg^{2+}. The dynamic molecules (*dyn*) are indicated in percent. L and H represent Low- and High-FRET states. (*G*) The relative abundance of dynamic traces from all single-traces under different Mg^{2+}. Mg^{2+} promotes flexibility and heterogeneity of riboTPP. All measurements were imaged in the absence of a TPP ligand.

FIGURE 3. Measurement of Mg^{2+} and TPP riboswitch interactions by ITC. ITC traces of riboTPP upon titration of 20 mM Mg^{2+} (*A*), 50 mM Mg^{2+} (*B*) and 80 mM (*C*). (*D*) The calculated thermodynamic parameters, Gibbs free energy (ΔG), Enthalpy (ΔH) and Entropy (ΔS).

FIGURE 4. Proposed model of Mg^{2+}-induced riboTPP folding. A high population of an open conformation (low-FRET ~ 0.4) without Mg^{2+}, likely corresponding to the *apo* of riboTPP. Entropy-driven folding of riboTPP to a compact and dynamic conformation (FRET ~ 0.7) upon the addition of Mg^{2+}, denoted as the folded state (F).